Crystal structures and kinetic analyses of *N*-acetylmannosamine-6-phosphate 2-epimerases from *Fusobacterium nucleatum* and *Vibrio cholerae*

Lavanyaa Manjunath,^{a,b} Sai Rohit Guntupalli,^{a,b} Michael J. Currie,^c Rachel A. North,^c Renwick C. J. Dobson,^{c,d} Vinod Nayak^a and Ramaswamy Subramanian^{a*}

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^aInstitute for Stem Cell Biology and Regenerative Medicine, NCBS, GKVK Campus, Bellary Road, Bangalore, Karnataka 560 065, India, ^bManipal Academy of Higher Education, Tiger Circle Road, Manipal, Karnataka 576 104, India, ^cBiomolecular Interaction Centre and School of Biological Sciences, University of Canterbury, Private Bag 4800, Christchurch, New Zealand, and ^dBio21 Molecular Science and Biotechnology Institute, Department of Biochemistry and Molecular Biology, University of Melbourne, Parkville, Victoria 3010, Australia. *Correspondence e-mail: ramas@instem.res.in

Sialic acids are nine-carbon sugars that are found abundantly on the cell surfaces of mammals as glycoprotein or glycolipid complexes. Several Gram-negative and Gram-positive bacteria have the ability to scavenge and catabolize sialic acids to use as a carbon source. This gives them an advantage in colonizing sialic acid-rich environments. The genes of the sialic acid catabolic pathway are generally present as the operon *nanAKE*. The third gene in the operon encodes the enzyme *N*-acetylmannosamine-6-phosphate 2-epimerase (NanE), which catalyzes the conversion of *N*-acetylmannosamine 6-phosphate to *N*-acetylglucosamine 6-phosphate, thus committing it to enter glycolysis. The NanE enzyme belongs to the isomerase class of enzymes possessing the triose phosphate isomerase (TIM) barrel fold. Here, comparative structural and functional characterizations of the NanE epimerases from two pathogenic Gram-negative bacteria, *Fusobacterium nucleatum* (Fn) and *Vibrio cholerae* (Vc), have been carried out. Structures of NanE from Vc (VcNanE) with and without ligand bound have been determined to 1.7 and 2.7 Å resolution, respectively. The structure of NanE from Fn (FnNanE) has been determined to 2.2 Å resolution. The enzymes show kinetic parameters that are consistent with those of *Clostridium perfringens* NanE. These studies allowed an evaluation of whether NanE may be a good drug target against these pathogenic bacteria.

1. Introduction

Sialic acids (specifically *N*-acetylneuraminic acid; Neu5Ac) are nine-carbon sugars that are ubiquitously found on mammalian cell surfaces as terminal sugars on glycoprotein and glycolipid conjugates (Varki, 1992). They play important roles in cell–cell recognition, interaction, adhesion and immunomodulatory processes (Varki, 1993, 2007; Vimr *et al.*, 2004). The respiratory, reproductive and gastrointestinal tracts of mammals are heavily sialylated (Vimr, 2013). Bacteria colonizing these environments have evolved mechanisms to sequester and use sialic acids as a source of energy by degrading them *via* a series of reactions to fructose 6-phosphate (Almagro-Moreno & Boyd, 2009a).

The enzymes of the catabolic pathway are present in a tightly regulated *nan-nag* operon (Vimr *et al.*, 2004; Vimr & Troy, 1985). These genes have been characterized in several Gram-positive and Gram-negative bacteria, and it has been shown that the catabolic pathway is essential for colonization

Table 1

Macromolecule-production information.

Source organism	<i>F. nucleatum</i> strain ATCC 25586	<i>V. cholerae</i>
Source of DNA	Synthetic DNA	Synthetic DNA
Expression vector	pET300/NT-DEST with N-terminal His tag	pET300/NT-DEST with N-terminal His tag
Expression host	<i>E. coli</i> Rosetta-gami 2(DE3)	<i>E. coli</i> BL21(DE3)
Sequence of the recombinant protein produced	MHHHHHITSLYKKAGFMNKILESIRGKLIVSCQALEDEPLHS SFIMGRMAYAAYSGGAAGRANTVEDIKEIKKNVSLPIIGI IKKVYNNSDVYITPTIKEVEDLINEGVQIIAIDATKRERPD RKDLKNFIAEIKEKYPNQLFMADISSVDEALYAEKIGFDIV GTTLVGYTDYTKNYKALEELKKVVKVVKIPVIAEGNIDTPL KAKKALEIGAFVAVVGGAITRPQOITKKFVDEMK	MHHHHHITSLYKKAGFMKNFLNIEELKRFLNGQTVVSIQPV TGSPLDKTDFFIVAMATAVEQAGAKALRIEVSNNVAASAAV TIPIIGIVKRDLPDSPVRITPFVSDVDGLANAGATVIAFDA TNRTREPESRERIAQAIKNTGCFAMADCSFTFEDGLWANSQGV EIVGSTLSGYVGDIPTVPDFQLVKAFSEAGFFTMAEGRYN TPELAAKAIESGAVAVTVGSALTRELVEVVTQWFNNATQAAGE RKCAH

and pathogenicity of the bacteria (Almagro-Moreno & Boyd, 2009b; Olson *et al.*, 2013). From the *nan* operon, the NanA, NanE and NanK enzymes are central to sialic acid breakdown. NanA is an *N*-acetylneuraminic acid lyase, which breaks down Neu5Ac to *N*-acetylmannosamine (ManNAc) and pyruvate. NanK is an *N*-acetylmannosamine kinase, which phosphorylates ManNAc to *N*-acetylmannosamine 6-phosphate (ManNAc-6-P), which is then epimerized to *N*-acetylglucosamine 6-phosphate (GlcNAc-6-P) by NanE (*N*-acetylmannosamine-6-phosphate 2-epimerase) (Fig. 1). A deacetylase and a deaminase convert GlcNAc-6-P to fructose 6-phosphate, which enters the glycolytic pathway (Plumbridge & Vimr, 1999).

NanE is an important enzyme in the sialic acid catabolic pathway as it commits ManNAc to glycolysis. Gene-deletion studies of NanE in *Vibrio cholerae* and *Staphylococcus aureus* have shown growth-inhibitory effects in the presence of Neu5Ac (Olson *et al.*, 2013). NanE is conserved in Gram-negative and Gram-positive bacteria and is not homologous in structure or mechanism to the mammalian UDP-*N*-acetylglucosamine 2-epimerases (North *et al.*, 2014; Pélissier *et al.*, 2014; Campbell *et al.*, 2000). This makes bacterial NanE an interesting target for potential antibacterial/antibiotic development.

To date, four structures of NanE are known: those from *Streptococcus pyogenes* (PDB entry 1xyx; Midwest Center for Structural Genomics, unpublished work), *S. aureus* (PDB entry 1y0e; Midwest Center for Structural Genomics, unpublished work), *Salmonella enterica* (SeNanE; PDB entries 3igs and 3q58; Center for Structural Genomics of Infectious Diseases, unpublished work) and *Clostridium perfringens* (CpNanE; PDB entries 4utt, 4utw and 4utu; Pélissier *et al.*, 2014). These epimerases belong to the TIM-barrel superfamily and have a common phosphate-binding domain. NanE belongs to the isomerase (EC 5.1.3.9) class of enzymes,

specifically epimerases whose substrates are carbohydrates and their derivatives. *C. perfringens* NanE has been studied biochemically and was reported to epimerize the sugar using a single lysine-mediated protonation and deprotonation (Pélissier *et al.*, 2014).

Bioinformatics analyses of *Fusobacterium nucleatum* (strain ATCC 25586) and *V. cholerae* have shown the presence of the *nanAKE* cluster of genes. *F. nucleatum* colonizes the oral cavity, intestines and reproductive tract, all of which are heavily sialylated. *F. nucleatum* is a major player in periodontitis and extra-oral infections of the skin, brain, vagina and uterus (Yoneda *et al.*, 2014). *V. cholerae* also colonizes the intestines, causing severe diarrhoea. In this paper, we report the crystal structures and kinetic analyses of the *N*-acetylmannosamine-6-phosphate 2-epimerases from *F. nucleatum* (FnNanE) and *V. cholerae* (VcNanE).

2. Materials and methods

2.1. Macromolecule production

2.1.1. Expression of FnNanE and VcNanE. FnNanE and VcNanE clones in the pET300/NT-DEST vector (Baird *et al.*, 2018) were transformed into *Escherichia coli* Rosetta-gami 2(DE3) and BL21(DE3) cells, respectively (Table 1). The cells were grown in Luria–Bertani broth containing ampicillin (100 µg ml⁻¹) and chloramphenicol (30 µg ml⁻¹) until the OD₆₀₀ reached 0.6. Expression was induced by the addition of 500 µM and 1 mM isopropyl β-D-1-thiogalactopyranoside for FnNanE and VcNanE, respectively. The cultures were grown at 293 K and 180 rev min⁻¹ for 16 h. The cells were harvested by centrifugation at 3220g for 30 min.

2.1.2. Purification of FnNanE. A Constant Systems cell disruptor was used for cell lysis. The cells were suspended in lysis buffer [50 mM Tris pH 8.0, 300 mM NaCl, 6% glycerol,

Figure 1

N-Acetylmannosamine-6-phosphate 2-epimerase catalyzes an inversion of stereochemistry at the C2 position, yielding *N*-acetylglucosamine 6-phosphate. This figure was produced using ChemDraw (CambridgeSoft Corporation, Cambridge, Massachusetts, USA).

Table 2
Crystallization information.

Protein	FnNanE	VcNanE
Method	Vapour diffusion, hanging drop	Vapour diffusion, hanging drop
Plate type	96-well plate	96-well plate
Temperature (K)	291	291
Protein concentration (mg ml ⁻¹)	18	28
Buffer composition	25 mM Tris pH 8.0, 50 mM NaCl, 6% glycerol, 10 mM BME	20 mM HEPES pH 7.5, 100 mM NaCl, 5 mM BME
Reservoir composition	0.1 M MES pH 5.2, 0.4 M MgCl ₂ , 22% PEG 3350	0.2 M malonate pH 5.0, 20% PEG 3350, 1 M malonate pH 7.0
Volume of drop	200 + 200 nl	500 + 500 nl
Volume of reservoir (μl)	100	100

10 mM β -mercaptoethanol (BME)]. A Roche cOmplete protease-inhibitor tablet was added to the cell suspension before lysis by two passes at 103 MPa. The cell debris was removed by centrifugation at 20 000g for 60 min at 277 K. The supernatant obtained by centrifugation was applied onto Ni-NTA beads (10 ml bed volume) equilibrated with buffer 1 (50 mM Tris pH 8.0, 300 mM NaCl, 20 mM imidazole, 6% glycerol, 10 mM BME). Affinity purification using the His tag was performed in batch mode. The bound protein was washed with 20 column volumes (CV) of buffer 1, 10 CV buffer 2 (50 mM Tris pH 8.0, 300 mM NaCl, 50 mM imidazole, 6% glycerol, 10 mM BME) and 10 CV buffer 3 (50 mM Tris pH 8.0, 300 mM NaCl, 100 mM imidazole, 6% glycerol, 10 mM BME), and was finally eluted with 2 CV buffer 4 (50 mM Tris pH 8.0, 300 mM NaCl, 300 mM imidazole, 6% glycerol, 10 mM BME). The eluate was concentrated to 8 mg ml⁻¹ using Amicon 30 kDa molecular-weight cutoff filter units and injected onto a Superdex 200 prep-grade column (GE Healthcare) pre-equilibrated with buffer 5 (25 mM Tris pH 8.0, 50 mM NaCl, 6% glycerol, 10 mM BME). 1 ml fractions were collected and run on SDS-PAGE to determine the purity of the sample before proceeding to crystallization trials. All purification steps were performed at 277 K. For ¹H NMR kinetics, the protein was purified into a buffer consisting of 25 mM Tris pH 8.0, 200 mM NaCl.

2.1.3. Purification of VcNanE. The cells were lysed by sonication (cycles of 5 s on and 15 s off at 40% amplitude for 40 min). The cell debris was removed by centrifugation at 20 000g for 60 min at 277 K. The supernatant obtained after centrifugation was applied onto a column of Ni-NTA beads (7 ml bed volume) equilibrated with buffer 1b (25 mM Tris pH 8.0, 300 mM NaCl, 20 mM imidazole, 5 mM BME). VcNanE was purified by batch binding chromatography. The protein was left to bind to the Ni-NTA beads for 1 h before washing with buffer 1b. The protein was eluted with 10 CV buffer 4b (25 mM Tris pH 8.0, 300 mM NaCl, 200 mM imidazole, 5 mM BME). The eluate was concentrated to 10 mg ml⁻¹ using Amicon 30 kDa molecular-weight cutoff filter units and injected onto a Superdex 200 prep-grade column (GE Healthcare) pre-equilibrated with buffer 5b (20 mM HEPES pH 7.5, 100 mM NaCl, 5 mM BME). The collected fractions were run on SDS-PAGE to determine the purity of the sample before proceeding to crystallization trials. All purification steps were performed at 277 K. For ¹H NMR kinetics, the protein was purified into a buffer consisting of 25 mM Tris pH 8.0, 200 mM NaCl.

2.2. Crystallization

The purified FnNanE and VcNanE proteins were concentrated to 18 and 28 mg ml⁻¹, respectively, using Amicon 30 kDa molecular-weight cutoff filter units. The concentrations were determined using both the Bradford assay and a NanoDrop 2000, using extinction coefficients of 13 410 and 14 130 M⁻¹ cm⁻¹ for FnNanE and VcNanE, respectively. The extinction coefficients were determined using the *ProtParam* tool from ExPASy (Gasteiger *et al.*, 2003). Initial crystallization trials were carried out using the commercially available screens PEG/Ion, PEG/Ion 2, Crystal Screen, Crystal Screen 2 (Hampton Research) and Wizard Classic 1 and 2 (Rigaku). FnNanE crystals diffracting to 2.3 Å resolution were obtained in a condition consisting of 0.1 M MES pH 5.2, 0.4 M MgCl₂, 22% PEG 3350 after refinement of the screening conditions (Table 2). The crystals were cryoprotected with reservoir buffer containing 30% PEG 400 and flash-cooled in liquid nitrogen. VcNanE crystals diffracting to 1.7 Å resolution were obtained in a condition consisting of 0.2 M malonate pH 5.0, 20% PEG 3350, 1 M malonate pH 7.0 (Table 2). Co-crystallization of VcNanE was performed by incubating 20 mM *N*-acetylglucosamine 6-phosphate with 33 mg ml⁻¹ purified VcNanE at 310 K for 30 min and crystallization was set up using the same conditions as for ligand-free VcNanE. The crystals of VcNanE were cryoprotected with reservoir buffer containing 30% PEG 400 and flash-cooled in liquid nitrogen.

2.3. Data collection and processing

Diffraction data were collected on the PROXIMA-1 beamline at the SOLEIL synchrotron. Data were processed with *XDS/XSCALE* (Kabsch, 2010) and scaled with *AIMLESS* from the *CCP4* suite (Evans & Murshudov, 2013). The data-processing statistics are provided in Table 3.

2.4. Structure solution and refinement

The phases of the FnNanE and VcNanE structures were obtained by molecular replacement using *Phaser-MR* in the *PHENIX* suite (Read, 2001; Adams *et al.*, 2011). The search models were monomeric polyalanine models of PDB entries 4utu (CpNanE; 58% sequence identity) and 3q58 (SeNanE; 51% sequence identity), respectively. The structure of VcNanE was used as a template to solve the structure of VcNanE with bound GlcNAc-6-P/ManNAc-6-P using *Phaser-MR*. Model building was carried out in *Coot* (Emsley *et al.*,

Table 3
Data collection and processing.

Data set	VcNanE–GlcNAc-6-P	VcNanE	FnNanE
Diffraction source	PROXIMA-1, SOLEIL	PROXIMA-1, SOLEIL	PROXIMA-1, SOLEIL
Wavelength (Å)	0.978	0.978	0.978
Temperature (K)	100	100	100
Detector	Dectris PILATUS 6M	Dectris PILATUS 6M	Dectris PILATUS 6M
Crystal-to-detector distance (mm)	346.9	384.9	441.3
Rotation range per image (°)	0.1	0.1	0.1
Total rotation range (°)	180	180	180
Exposure time per image (s)	0.1	0.1	0.1
Space group	$P2_12_12_1$	$P2_12_12_1$	$I222$
<i>a</i> , <i>b</i> , <i>c</i> (Å)	48.96, 70.65, 149.36	49.32, 70.42, 150.40	47.52, 75.35, 135.88
α , β , γ (°)	90, 90, 90	90, 90, 90	90, 90, 90
Mosaicity (°)	0.55	0.09	0.14
Resolution range (Å)	40.94–2.66 (2.75–2.66)	46.87–1.70 (1.73–1.70)	44.85–2.21 (2.27–2.21)
Total No. of reflections	89562 (10022)	365016 (13639)	80920 (6997)
No. of unique reflections	15416 (1900)	58493 (2962)	12687 (1086)
Multiplicity	5.8 (5.3)	6.2 (4.6)	6.4 (6.4)
Completeness (%)	99 (94.3)	100 (100)	100 (100)
Mean $I/\sigma(I)$	8.7 (2.4)	15.2 (3.7)	13.5 (2.8)
Wilson <i>B</i> factor (Å ²)	43.5	17.8	49.6
R_{meas}	0.152 (0.555)	0.097 (0.473)	0.076 (0.761)
$R_{\text{p.i.m.}}$	0.062 (0.230)	0.038 (0.217)	0.030 (0.297)
$CC_{1/2}$	0.99 (0.92)	0.99 (0.91)	0.99 (0.78)

Table 4
Structure-solution and refinement statistics.

Values in parentheses are for the outer shell.

Data set	VcNanE– GlcNAc-6-P	VcNanE	FnNanE
Reflections used in refinement	15355 (1423)	58402 (5699)	12678 (1242)
Reflections used for R_{free}	736 (79)	2970 (296)	644 (54)
R_{work}	0.23 (0.30)	0.18 (0.21)	0.18 (0.28)
R_{free}	0.28 (0.36)	0.21 (0.26)	0.23 (0.37)
No. of non-H atoms			
Total	3429	3872	1776
Macromolecules	3359	3474	1745
Ligands	66	47	2
Protein residues	453	464	225
R.m.s.d., bonds (Å)	0.005	0.007	0.009
R.m.s.d., bonds (°)	1.1	1.1	0.9
Ramachandran favoured (%)	96	97	96
Ramachandran allowed (%)	3.8	2.4	3.6
Ramachandran outliers (%)	0.22	0.21	0.45
Rotamer outliers (%)	0.56	0.81	0.53
Clashscore	3.96	4.26	6.72
Average <i>B</i> factor (Å ²)			
Overall	45.4	22.2	66.5
Macromolecules	45.3	21.5	66.7
Ligands	51.8	30.1	80.8
Solvent	41.3	28.7	54.6

2010) and the structures were iteratively refined using the *PHENIX* suite. Water molecules were automatically added during the process of refinement, but were manually checked for density fit to both $2|F_o| - |F_c|$ and $|F_o| - |F_c|$ maps and for hydrogen bonding. The structure-solution and refinement statistics are provided in Table 4. The overall structure is shown in Fig. 2 and details of the active site with ligand bound are shown in Fig. 3.

2.5. Enzyme kinetics

2.5.1. ¹H NMR assay. When collecting initial rate data using the NMR assay, 100 nM VcNanE was added to a 500 µl

reaction mixture containing various concentrations of ManNAc-6-P and 4 mM α -alanine (internal standard). Stock solutions of ManNAc-6-P and α -alanine were prepared in a D₂O assay buffer consisting of 25 mM Tris pH 8.0, 200 mM NaCl at 298 K. The ¹H NMR spectra were recorded at 298 K on a Bruker Avance III 600 spectrometer for 10 min after adding VcNanE to the reaction mixture. We observed that the NMR signal of the acetyl CH₃ group of ManNAc-6-P had two singlet peaks corresponding to the α - and β -anomers at 2.040 and 2.002 p.p.m., respectively. The rate of the reaction was obtained by calculating the rate of disappearance of the ManNAc-6-P peak (Fig. 4*a*, inset). The initial and final concentrations of ManNAc-6-P were calculated by integrating the peak area of the acetyl CH₃ signal of ManNAc-6-P with respect to the acetyl CH₃ signal of α -alanine (Fig. 4*a*). The kinetics experiments were carried out in duplicate. A nonlinear regression fitting the Michaelis–Menten equation (*GraphPad Prism* v.6.0) was used to calculate the kinetic parameters V_{max} , K_m and k_{cat} .

2.5.2. Coupled assay. A novel coupled assay was performed by coupling NanE with the subsequent two enzymes in the sialic acid catabolism pathway, *N*-acetylglucosamine-6-phosphate deacetylase (NagA) and glucosamine-6-phosphate deaminase (NagB) (both purified in-house; the enzymes were from the methicillin-resistant *S. aureus* strain USA300), phosphoglucosomerase (PGI; Sigma–Aldrich) and glucose-6-phosphate dehydrogenase (G6PDH; Sigma–Aldrich). The reduction of NADP⁺ to NADPH was measured at 340 nm (Fig. 4*b*) using a Cary 300 UV–visible spectrophotometer (Agilent Technologies). The experiments were carried out at 298 K in a reaction mixture consisting of 100 mM Tris pH 8.0, 1 mM NADP⁺, 5 mM MgCl₂. The reaction mixture (1 ml) contained various concentrations of ManNAc-6-P (0.15625–5 mM) and excess quantities of the coupling enzymes, to which 10 nM FnNanE or 100 nM VcNanE were added to initiate the

reaction. The change in absorbance at 340 nm was converted to the change in substrate concentration using the Beer–

Lambert law and an extinction coefficient of $6220\text{ M}^{-1}\text{ cm}^{-1}$ (NADPH). The kinetics experiments were carried out in

Figure 2

Overall structure of NanE. (a) Cartoon representation of the VcNanE dimer with individually coloured monomer secondary structures. Helices are numbered $\alpha 0$ – $\alpha 8$ and sheets $\beta 1$ – $\beta 8$. (b) Orthogonal view showing the helix-swapped dimeric assembly of VcNanE. The active sites are on the opposite side of the faces of the monomers. Figures were produced using *PyMOL*.

Figure 3

Electron densities for ligand and active-site interactions. Electron-density ($2F_o - F_c$) maps contoured at 1.0σ showing density for ManNAc-6-P (a) and GlcNAc-6-P (b). Both GlcNAc-6-P and ManNAc-6-P could be modelled into the ligand density at the active site. (c) Ligand–active site interactions are retained with both ManNAc-6-P (depicted in cyan) and GlcNAc-6-P (depicted in yellow). Only hydrogen bonds made between ManNAc-6-P and VcNanE (depicted as green sticks; sticks in blue are from the VcNanE–GlcNAc-6-P model) are depicted here with dashed black lines for clarity. Distances are labelled in black for ManNAc-6-P and in blue and red (electrostatic interactions) for GlcNAc-6-P. Chiral centres are labelled C2–C5. The change in chirality at the C2 centre is clearly visible. Figures were produced using *PyMOL*.

duplicate. A nonlinear regression fitting the Michaelis–Menten equation (*GraphPad Prism* v.6.0) was used to calculate the kinetic parameters $V_{\max}$, K_m and k_{cat} .

3. Results and discussion

3.1. Data processing and structure solution

3.1.1. Structure of VcNanE. The structure of VcNanE was refined using data to 1.7 Å resolution. Details of the data-

collection and processing statistics can be found in Table 3. VcNanE crystallized in space group $P2_12_12_1$, with two molecules in the asymmetric unit forming the biologically observed dimer. The Matthews coefficient is $2.61 \text{ Å}^3 \text{ Da}^{-1}$, with 52.94% solvent content. The first 18 amino acids (inclusive of the purification tag derived from the pET300/NT-DEST vector) and the last two amino acids of chains *A* and *B* could not be modelled in the structure. The final structure showed good geometry with one Ramachandran outlier, three molecules of malonate (MLI), two molecules of ethylene glycol (EDO), two

Figure 4 Real-time ¹H NMR kinetics and coupled-assay kinetics of VcNanE and FnNanE activity. (a) ¹H NMR spectra signals for the acetyl CH₃ groups of ManNAc-6-P (red) and GlcNAc-6-P (blue) and the internal standard α-L-alanine (labelled). Inset: time course of ¹H NMR spectra showing the depletion of ManNAc-6-P and the increase in GlcNAc-6-P after the addition of VcNanE to the reaction mixture. (b) Multi-enzyme coupled assay. The reaction rate was measured spectrophotometrically at 340 nm by monitoring the reduction of NADP⁺ to NADPH. Coupling enzymes are depicted in green. (c) ¹H NMR kinetics of VcNanE. (d) Coupled-assay kinetics of VcNanE. (e) Global fit of the VcNanE coupled assay and ¹H NMR kinetics. (f) Coupled-assay kinetics of FnNanE. All data were fitted to the Michaelis–Menten equation $V_0 = V_{\max}[S]/K_m + [S]$ using *GraphPad Prism* v.6.0.

molecules of polyethylene glycol (PEG) and 351 water molecules. The R factor and R_{free} for the final model are 0.18 and 0.21, respectively.

3.1.2. Ligand-bound structure of VcNanE. VcNanE was co-crystallized with GlcNAc-6-P. The diffraction data were processed and scaled to a resolution of 2.7 Å with 99% completeness. Details of the data-collection and processing statistics can be found in Table 3. Ligand-bound VcNanE also crystallized in space group $P2_12_12_1$ with two molecules in the asymmetric unit. The Matthews coefficient is $2.32 \text{ Å}^3 \text{ Da}^{-1}$, with 47% solvent content.

The structure of VcNanE was used as a homology model to solve the structure of ligand-bound VcNanE by molecular replacement. The model was built in *Coot* and refined using *PHENIX*. After ten refinement cycles the $F_o - F_c$ density clearly showed the binding site for the ligand (Supplementary Fig. S2). A composite OMIT map also showed unambiguous density for the ligand. We modelled the structure with GlcNAc-6-P, yielding the structure VcNanE–GlcNAc-6-P (Table 4). We also modelled the structure with ManNAc-6-P, yielding the structure VcNanE–ManNAc-6-P. In solution, the enzyme catalyzes the formation of ManNAc-6-P from GlcNAc-6-P. After modelling the ligand, several rounds of refinement with NCS, TLS and rotamer restraints were carried out. The first 21 amino acids and 20 amino acids (inclusive of the purification tag derived from the pET300/NT-DEST vector) of chains *A* and *B*, respectively, could not be modelled in the structure. In both chains *A* and *B* density for amino acids 160–164 was not observed. The last six amino acids and five amino acids of chains *A* and *B*, respectively, could also not be modelled in the structure. The final structure showed good geometry, with three Ramachandran outliers, one molecule of ethanediol, one molecule of triethylene glycol, two molecules of di(hydroxyethyl)ether and four waters. The R factor and R_{free} for the final models were 0.23 and 0.28, respectively, for VcNanE–GlcNAc-6-P and were 0.23 and 0.28, respectively, for VcNanE–ManNAc-6-P.

3.1.3. Structure of FnNanE. The structure of FnNanE was determined to 2.2 Å resolution. Details of the data-collection and processing statistics can be found in Table 3. FnNanE crystallized in space group $I222$ with one molecule in the asymmetric unit. The Matthews coefficient is $2.43 \text{ Å}^3 \text{ Da}^{-1}$, with 49.46% solvent content. A monomer model of CpNanE (PDB entry 4utu) was used as a homology model for molecular replacement. Only the first 15 amino acids at the N-terminus could not be modelled in the structure. The final structure showed good geometry with one Ramachandran outlier, two chloride ions and 30 water molecules. The R factor and R_{free} for the final model were 0.20 and 0.23, respectively.

3.2. Overall structures of VcNanE and FnNanE

3.2.1. TIM-barrel protein with helix swapping in a dimeric assembly. Similar to other structures of NanE, FnNanE and VcNanE are composed of eight α -helices and eight β -strands forming a modified triosephosphate isomerase (TIM) barrel (Fig. 2a). The eighth helix stacks against the β -sheet of another

molecule, thus forming a helix-swapped dimer (Fig. 2b). Each subunit has an extra short helix at the N-terminus which caps one side of the barrel. The active-site pocket is relatively solvent-accessible and is formed by the $\beta 2$ – $\alpha 2$ and the $\beta 3$ – $\alpha 4$ loops (Fig. 2b). The two active sites are on opposite faces of the two monomers and are at least 30 Å apart from each other (Fig. 2b, Supplementary Fig. S1a). The buried surface area is 4680 Å^2 between the monomers that form the VcNanE dimer. While there is only one subunit in the asymmetric unit of FnNanE, the biological oligomeric state is a dimer. The two symmetry-related monomers that form the dimer bury 4927 Å^2 (Krissinel & Henrick, 2007; Evgeny, 2010).

3.2.2. Structural comparison: ligand binding does not affect the tertiary structure. The FnNanE and VcNanE structures are very similar to each other, with an r.m.s.d. of 1.6 Å for 220 C^α atoms. A pairwise superposition of the structures of FnNanE and VcNanE with other homologues (PDB entries 3igs, 3q58, 1xyx, 1y0e and 4utw) also shows that the NanE enzymes are not structurally different from each other (r.m.s.d. in the range 1.5–1.7 Å; Krissinel & Henrick, 2004). The ligand-bound and ligand-free structures of VcNanE have an r.m.s.d. of 0.45 Å for 450 C^α atoms (from the dimer). The positions of the side chains of the active-site residues between the ligand-bound and ligand-free structures also do not show significant differences. A pairwise superposition of the ligand-bound VcNanE structure and ligand-free SpNanE (PDB entry 1xyx; 36% sequence identity) gave an r.m.s.d. of 1.4 Å with 209 aligned residues. The relative orientations of the active-site amino acids (VcNanE) remain unchanged when compared with those in SpNanE. Only the side chain of Arg220 of SpNanE shows flexibility (Supplementary Fig. S3). Taken together, these observations indicate that the active-site architecture is rigid and the presence of the ligand does not alter the tertiary structure of the protein.

3.2.3. Substrate-binding site. The crystal structure of VcNanE shows unambiguous density for malonate (MLI) at the ligand-binding site. The pocket in which malonate binds is the same as that for the substrate (Supplementary Figs. S1a and S1b). Malonate is a component of the crystallization conditions for VcNanE crystals and forms hydrogen bonds to three water molecules and the amide N atoms of Gly209, Ser210 and Arg188 in the active site (Supplementary Fig. S1c).

The active site of VcNanE crystals soaked with GlcNAc-6-P shows density for an open-chain sugar phosphate in the active site (Supplementary Fig. S2). Péliissier and coworkers also showed that GlcNAc-6-P (open-chain form) was bound in the active site when CpNanE crystals were soaked with ManNAc-6-P (PDB entry 4utw) and that ManNAc-6-P was bound in the Lys69 mutant (PDB entry 4utu) (Péliissier *et al.*, 2014). In solution, the enzyme also catalyzes the reverse reaction (GlcNAc-6-P to ManNAc-6-P), although it favours the formation of GlcNAc-6-P (Ringenberg *et al.*, 2003). Therefore, ManNAc-6-P and GlcNAc-6-P were both modelled into the density (Figs. 3a and 3b). It was not clear from the electron density at the active site whether the bound ligand was ManNAc-6-P or GlcNAc-6-P. One possible way of carrying out refinement would be to model both in the same active site with

partial occupancy. This would correspond to an assumption that both the substrate and the product are present in the binding pocket. Since there is no evidence to justify the presence of a mixed population, both ManNAc-6-P and GlcNAc-6-P were fitted independently and both models were refined against the same data set. While the tables only represent the fit to the structure with GlcNAc-6-P, we have

submitted coordinates to the PDB for both refinement results (PDB entries 5zjp and 5zjn for the complexes with GlcNAc-6-P and ManNAc-6-P, respectively). The structures converge to similar final refinement statistics.

The O atoms of the phosphate group of ManNAc-6-P and GlcNAc-6-P hydrogen-bond to the main-chain amide N atoms of Gly209, Arg188 and Ser210 (Fig. 3c). The O3 and O4

Table 5
Kinetic parameters of FnNanE and VcNanE.

	$V_{\max}$ ($\mu\text{mol min}^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1} \text{mM}^{-1}$)
VcNanE coupled assay	0.069 ± 0.004	3.5 ± 0.5	28.0	8.0
VcNanE NMR	0.060 ± 0.003	2.4 ± 0.3	24.5	10.0
Global fit of VcNanE coupled assay and NMR	0.063 ± 0.002	2.8 ± 0.2	25.4	9.0
FnNanE coupled assay	0.025 ± 0.0008	1.0 ± 0.1	41.0	41.0

Table 6
Comparison of the K_m values of different sugar epimerases.

Epimerase	K_m (mM)
CpNanE	4.7
<i>R. albus</i> cellobiose 2-epimerase	13
Human UDP- <i>N</i> -acetylglucosamine 2-epimerase	0.2
<i>E. coli</i> galactose mutarotase	4
FnNanE	1
VcNanE	4

Figure 5
Sequence-based alignment of FnNanE and VcNanE with other NanEs with structures in the PDB. FnNanE has 58% identity to CpNanE (PDB entry 4utt), 33% identity to VcNanE and 37, 50 and 53% identity to SeNanE (PDB entry 3q58), SpNanE (PDB entry 1y0e) and SaNanE (PDB entry 1xyx), respectively. VcNanE has 51% identity to SeNanE and 34, 30 and 35% identity to SpNanE, SaNanE and CpNanE, respectively. Strictly conserved active-site residues are marked with a blue star; the catalytic lysine residue is conserved across all species and is marked with a green star. Nonconserved active-site residues are marked with a pink star. Alignment was performed using *MultAlin* and the figure was generated using *ESPr3*.

Table 7

Ligand–active site interactions in different NanE structures.

Interactions with the peptide bonds of the main chain are shown in bold.

	FvNanE (this study)	VcNanE (this study)	CpNanE (PDB entry 4utu)	SaNanE (PDB entry 1y0e)	SpNanE (PDB entry 1yxy)	SeNanE (PDB entry 3q58)
O1 PO ₄ ³⁻	Gly206	Gly209	Gly203	Gly203	Gly215	Gly207
O2 PO ₄ ³⁻	Gly207	Ser210	Gly204	Gly204	Gly216	Ser208
O3 PO ₄ ³⁻	Asn185	Arg188	Arg182	Asn182	Lys194	Arg186
C1 aldehyde	Gln17	Gln24	Gln14	Gln11	Gln23	Gln22
C3 hydroxyl	—	Arg214	Solvent	—	—	—
C4 hydroxyl	Arg211	Arg214	Arg208	Arg208	Arg220	Arg212
C5 hydroxyl	Glu183	Glu188	Glu180	Glu180	Glu192	Glu184
Acetyl carbonyl	Tyr78	—	Tyr75	—	—	—

hydroxyl groups of the sugar hydrogen-bond to the guanidinium group of Arg214 and the O5 hydroxyl forms a hydrogen bond to the carboxyl group of Glu186. The carbonyl (O1) of the aldehyde (C1 aldehyde) group hydrogen-bonds to the side-chain amide of Gln24 and the side-chain guanidinium group of Arg53, and the carbonyl of the *N*-acetyl group could potentially hydrogen-bond to Arg85 through a solvent molecule.

A comparison of the models of the ManNAc-6-P-bound and GlcNAc-6-P-bound forms reveals that most of the active-site interactions are retained, but with a few changes to accommodate the inversion of stereochemistry at the C2 stereocentre. The changes are in the O1 (C1 aldehyde), O3 and O4 hydroxyl groups and the C2 positions of the two epimers. In the GlcNAc-6-P-bound form, the hydrogen-bonding distances for the phosphate group, O5 (with Glu188), O4 and O3 (with Arg214) hydroxyl groups all change by 0.2 Å. The largest change in hydrogen-bonding distance is seen between the C1 aldehyde and Arg53; the difference is 0.6 Å. In addition, the ϵ -amino group of the catalytic residue Lys76 is positioned at a distance of 4.1 Å from the C2 atom of GlcNAc-6-P, while it is at a distance of 3.3 Å in the ManNAc-6-P-bound structure. The reported lysine N^ε–C2 distance for the CpNanE structure is 2.5 Å (Pélissier *et al.*, 2014).

3.3. Enzyme kinetics

Coupled assays as well as real-time ¹H NMR were carried out to assess the kinetic parameters of FvNanE and VcNanE. For FvNanE we only performed the coupled assay, while for VcNanE both the coupled assay and ¹H NMR were performed. The data obtained for VcNanE using both methods were consistent with Michael–Menten kinetics. Also, the kinetic parameters obtained using both methods were in a similar range (Figs. 4c–4e, Table 5). The FvNanE data also follow Michaelis–Menten kinetics (Fig. 4f). The apparent *K_m* with ManNAc-6-P was ~1 and ~3 mM for FvNanE and VcNanE, respectively (Table 5). The *k_{cat}* was 41 s^{−1} and ~26 s^{−1} and the *k_{cat}*/*K_m* was 41 and 9 s^{−1} mM^{−1} for FvNanE and VcNanE, respectively (Table 5). The reported *K_m* values for *C. perfringens* NanE (Pélissier *et al.*, 2014), *Ruminococcus albus* cellobiose 2-epimerase, human UDP-*N*-acetylglucosamine 2-epimerase and *E. coli* galactose mutarotase (Beebe *et al.*, 2003) are also in the low-millimolar range (Table 6).

Sequence-based multiple-alignment analyses show that the catalytic lysine residue is conserved across species (Fig. 5). The structures of VcNanE and FvNanE are similar to those of other *N*-acetylmannosamine-6-phosphate 2-epimerases solved to date. The amino acids that interact with the ligand are well conserved, with a few exceptions (Table 7). The published CpNanE structure, the three unpublished structures determined by structural genomics consortia and the structures described here all show that the active site is well conserved (Supplementary Fig. S3). Therefore, an inhibitor that inhibits one enzyme should have the ability to inhibit many other enzymes (from a variety of pathogens), making NanE a target for structure-assisted drug discovery.

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